

# Loosening of DNA/Polycation Complexes by Synthetic Polyampholyte to Improve the Transcription Efficiency: Effect of Charge Balance in the Polyampholyte

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**ABSTRACT** High mobility group proteins are amphoteric nuclear proteins that are known to unfold chromatin to stimulate transcription. To mimic their structures, we synthesized the novel polyethylene glycol (PEG) derivatives, PEG-ACs, consisting of both amino- and carboxyl-pendants in various ratios, and their loosening and transcription-improving activity on the DNA complex was examined. Fluorescence anisotropy measurement revealed that anionic PEG-ACs with more carboxyls than amines could efficiently loosen the DNA/polyethyleneimine complex. Those anionic PEG-ACs showing a loosening effect on the DNA complex evidently increased the transcription rate to >20 times higher than that of the original complex, probably owing to the facilitated approach of transcriptional factors to the DNA segments in the loosened complexes. The complexes with anionic PEG-ACs also showed improved transgene expression level on the cultured cells, indicating the effectiveness of improving transcriptional activity to attain a high extragenic expression by the plasmid complex. The loosening mechanism of DNA/polycation complexes was investigated with a simplified model via Monte Carlo simulation to discern the difference in the presence of cationic polyampholytes, anionic polyampholytes, and polyanions.

## INTRODUCTION

A great deal of effort has been focused on developing highly efficient nonviral vector systems as a safer alternative to viruses. Low transfection efficiency is, however, still a major problem of the synthetic vectors. Low gene expression by nonviral vectors can be attributed to low efficiency in the delivery to the target tissue or cells, uptake by the cells, escape from the endocytotic vesicles, and translocation to the cell nucleus (1,2). Recently, it was revealed that low transcription efficiency of the plasmid/polycation complexes also strongly interfered with effective gene expression by nonviral vector systems (3).

Various attempts have been made to improve nonviral vector systems to attain high gene expression, of which some examples are polyanion-coating to avoid adverse interaction with biocomponents (4,5), introduction of ligands to adhere to the target cells (6–8), and proton sponge function for efficient endosomal escape (9). However, only a few reports have been published about attempts to enhance the transcriptional efficiency (10). Our attention has been focused on devising an effective method to loosen the plasmid/polycation complexes to facilitate the access of transcription factors.

High mobility group proteins are amphoteric nuclear proteins, and have been reported to stimulate transcription by unfolding the chromatin structure (11,12). Recently, we

successfully synthesized a novel amphoteric polyethylene glycol (PEG) derivative bearing both amino- and carboxyl-pendants. The transcriptional enhancing activity of the polyampholyte was evaluated using  $\gamma$ -amino naphthalene sulfonic acid uridine 5'-triphosphate (UTP) by the fluorescence method (13).

It was noted that the consumption of the UTP analog with a plasmid/polyethyleneimine (PEI) binary complex was much lower than with a naked plasmid DNA. A surprising finding was that adding amphoteric PEG derivative (PEG-AC) to the complexes significantly increased the transcription activity of the plasmid/PEI binary complex. Moreover, the polyampholyte showed higher enhancing ability than the simple anionic PEG derivative without amino groups. The polyampholyte thus seems to have a high potential to loosen the DNA/PEI complexes, and activate the transcription (14).

To better understand the above experimental findings, a simple model was investigated in our previous work to reveal the possible mechanisms by which polyampholytes and polyanions assist the dissociation of DNA/polycation complexes (15). The model, modified from the cell model, represented a local region of a segment in a long DNA chain. We considered a DNA/polycation complex mixing with a polyampholyte of zero net charge and with a polyanion via electrostatic interactions at the Debye-Hückel level. A short rigid DNA segment in a long DNA chain was modeled as a charged cylinder, and the other ionic polymers were treated as charged shish-kebab chains. The results of Monte Carlo simulation showed that the polycation may be dissociated from DNA by polyanions when the linear charge density of the polyanions becomes large enough to weaken the adsorption of a polycation on the like-charge DNA. On

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the other hand, dipolar polyampholytes may enhance transcriptional activity through a different mechanism with electrostatic bridging to connect the DNA and polycation with their oppositely charged segments. Such a mechanism opens up space near DNA and may facilitate the access of transcriptional factors to DNA.

These two mechanisms, dipolar electrostatic bridging of polyampholytes and competitive adsorption of polyanions against DNA, govern the complex-loosening phenomena, which lay the physics ground for designing the optimum structure and charge balance of the polyampholyte to achieve the highest transcription efficiency. In this study, several PEG-ACs with varied ratios of amino- to carboxyl- side chains ( $\text{NH}_2/\text{COOH}$  ratio) were synthesized and explored for their loosening ability on the DNA/polycation complexes, and their transcription enhancing activity was examined. The loosening mechanism was also investigated by extending the model in Shew et al. (15) to discern the subtle difference in the roles played by cationic polyampholytes, anionic polyampholytes, and polyanions in the transcriptional activity through Monte Carlo simulation.

## METHODS AND MATERIALS

### Methods

Plasmid DNA (7.0 kbp) containing the firefly luciferase gene under control of a cytomegalovirus promoter was a kind gift from Prof. S. Kawakami and Prof. M. Hashida (Kyoto University, Kyoto, Japan). It was amplified in *Escherichia coli* and purified with a QIAGEN plasmid mega kit. *E. coli* RNA polymerase holoenzyme was obtained from Epicentre Technologies (Madison, WI). The RiboGreen RNA Quantitation Kit and deoxyribonuclease I (DNase I) from bovine pancreas were purchased from Invitrogen (Carlsbad, CA). ATP, CTP, GTP, and UTP were obtained as lithium salt from Roche Diagnostics (Indianapolis, IN). Linear PEI (molecular weight 25,000) was purchased from Polysciences (Warrington, PA). Salmon sperm DNA (30–45 kbp), and 4'-diamidinophenylindol (DAPI) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Poly(allyl glycidyl ether-co-ethylene oxide) (molecular weight 8940) synthesized as described before (16) was kindly given by NOF Corporation (Tokyo, Japan). Statistical analysis for comparison between groups was performed with a Student's *t*-test (two-tailed).

### Synthesis of amphoteric PEG derivatives

PEG-ACs having amino- and carboxyl-pendants at amine/carboxyl ratios ( $\text{NH}_2/\text{COOH}$ ) of 0:100, 11:89, 18:82, 32:68, and 37:63 were synthesized as follows. PEG-ACs were directly prepared by the reaction of poly(allyl glycidyl ether-co-ethylene oxide) in the mixed solution of aminoethanethiol hydrochloride and mercaptopropionic acid. Typically, to the mixed solution of aminoethanethiol hydrochloride (285 mg) and mercaptopropionic acid (1060 mg) in methanol (1 mL) was added 500 mg of poly(allyl glycidyl ether-co-ethylene oxide) (17.7 allyl pendants/polymer; molecular weight 7060) dissolved in 1.5 mL of methanol. After standing at 40°C for 3 days, the solution was diluted with water and dialyzed against running water overnight and against distilled water for 1 more day. After gel-filtration with a Sephadex G50 and ion-exchange with a sulfonate-type resin, the product was freeze-dried to a colorless thick syrup (548 mg). The molar  $\text{NH}_2/\text{COOH}$  ratio in the polymer was determined to be 18:82 by the  $^1\text{H}$  NMR spectrum of its acetylated derivative. Other PEG-ACs with different  $\text{NH}_2/\text{COOH}$  ratios were obtained by changing the ratio of aminoethanethiol to

mercaptopropionic acid in the reaction solution, unless the molar amount of aminoethanethiol did not exceed that of mercaptopropionic acid.

Reaction of poly(allyl glycidyl ether-co-ethylene oxide) with the mixture of excess aminoethanethiol over mercaptopropionic acid was not successful, probably due to the acidic conditions of the mixture. PEG-ACs with an  $\text{NH}_2/\text{COOH}$  ratio of 60:40 or 94:6 were then synthesized by the two-step reaction. Typically, 100 mg of poly(allyl glycidyl ether-co-ethylene oxide) was first treated with excess aminoethanethiol hydrochloride (200 mg) for 5 min at 40°C, then purified and treated with an extra amount of mercaptopropionic acid (500 mg) for 4 days at 40–50°C. Gel filtration and lyophilization gave 42.8 mg of the polymer with  $\text{NH}_2/\text{COOH} = 60:40$ . PEG-AC with an  $\text{NH}_2/\text{COOH}$  ratio of 100:0 was synthesized as previously reported (16).

### Measurement of $\zeta$ -potential and size of the complex

Samples were prepared by mixing the aqueous solutions of plasmid (1.25  $\mu\text{g}$  in 12.5  $\mu\text{L}$ ) and linear PEI (1.30  $\mu\text{g}$  in 12.5  $\mu\text{L}$ ) and then adding aqueous PEG-AC solution (30.6  $\mu\text{g}$  in 25  $\mu\text{L}$ ). This mixing weight ratio corresponds to  $\text{P/N/COOH} = 1:8:16$  in mole for PEG-AC(0:100). The suspension was diluted with water (950  $\mu\text{L}$ ) and analyzed for  $\zeta$ -potential and diameter by a MALVERN Zetasizer Nano ZS.

Fluorescence spectrum and anisotropy were measured with a spectrofluorometer (FP-6500, JASCO, Tokyo, Japan) equipped with a fluorescence polarization measurement unit, APH-103.

### Transcription procedure

The plasmid complex suspension was prepared by mixing the aqueous solutions of plasmid (1  $\mu\text{g}$  in 10  $\mu\text{L}$ ) with linear PEI (1.04  $\mu\text{g}$  in 10  $\mu\text{L}$ ) and PEG-AC (24.5  $\mu\text{g}$  in 20  $\mu\text{L}$ ). A mixture of 1 M Tris-HCl (0.9  $\mu\text{L}$ , pH 7.5), 3 M KCl (3  $\mu\text{L}$ ), 1 M  $\text{MgCl}_2$  (0.45  $\mu\text{L}$ ), 10 mM each ATP, CTP, GTP, and UTP (each 0.9  $\mu\text{L}$ ), 100 mM DTT (1.8  $\mu\text{L}$ ), and 50.25  $\mu\text{L}$  of water was added to the plasmid complex suspension prepared above. After incubation for 30 min at 37°C, *E. coli* RNA polymerase (1 U/ $\mu\text{L}$ ; 2  $\mu\text{L}$ ) was added, and the samples were kept at 37°C for 2 h with occasional vortexing. To the reaction mixture was added the 10 mM Tris-HCl (200  $\mu\text{L}$ , pH 8.0) saturated with phenol and containing 1 mM EDTA and 300  $\mu\text{L}$  of water. After vortexing, it was centrifuged for 5 min at 4°C, and the supernatant was mixed with glycogen (20 mg/mL; 2  $\mu\text{L}$ ) and 3 M sodium acetate (40  $\mu\text{L}$ ) and chilled ethanol (1 mL). After storage for 30 min at  $-80^\circ\text{C}$ , it was again centrifuged for 20 min at 4°C. The precipitated RNA was washed with cold 70% ethanol and air-dried. The RNA was then dissolved in 0.5 mL of water and added to a mixture of 40 mM Tris-HCl buffer (57  $\mu\text{L}$ , pH 7.9) containing 10 mM NaCl, 6 mM  $\text{MgCl}_2$ , and 10 mM  $\text{CaCl}_2$ , bovine serum albumin (2 mg/mL; 6  $\mu\text{L}$ ) and DNase (1 U/mL; 1  $\mu\text{L}$ ). After standing 1 h at 37°C, RiboGreen solution in Tris-HCl buffer (564  $\mu\text{L}$ , pH 8.0) containing 1 mM EDTA was added, and RNA was quantified by a fluorescence spectrometer ( $\text{Ex} = 485 \text{ nm}$ ;  $\text{Em} = 530 \text{ nm}$ ).

### Fluorescence anisotropy

The loosening effect of the PEG-ACs on the DNA/PEI complex was evaluated by fluorescence anisotropy assay for DAPI associated with DNA. To an aqueous solution of salmon sperm DNA (5  $\mu\text{g}$  in 12.5  $\mu\text{L}$ ) were added mercaptoethanol (12.5  $\mu\text{L}$ ), DAPI (50  $\mu\text{M}$  in 25  $\mu\text{L}$ ), and phosphate-buffered saline (PBS; 950  $\mu\text{L}$ ), and the anisotropy for DAPI was measured. PEI (5.2  $\mu\text{g}$  in 50  $\mu\text{L}$ ) was then added to the plasmid solution at  $\text{N/P} = 8$ , and the fluorescence anisotropy was again measured. PEG-AC (122.5  $\mu\text{g}$  in 100  $\mu\text{L}$ ) was added to the DNA complex, and the anisotropy was monitored. Complexes prepared by adding PEG-ACs before the PEI were analyzed in a similar way.

### In vitro transfection procedure

Plasmid/PEI/PEG-AC ternary complex was prepared by mixing the solution of plasmid (1.25  $\mu\text{g}$  in 12.5  $\mu\text{L}$  water) and linear PEI (1.3  $\mu\text{g}$  in 12.5  $\mu\text{L}$  water), then adding PEG-AC (30.6  $\mu\text{g}$  in 25  $\mu\text{L}$  water). After the complex

was allowed to stand for 30 min, double-concentrated PBS (50  $\mu$ L) was added. B16 cells, a mouse melanoma cell line, or Chinese hamster ovary (CHO) cells were seeded into 24-well multiplates at  $\sim 2.5 \times 10^4$  cells/well, and grown for 2 days in D-MEM medium containing 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin sulfate (0.1 mg/mL). The primary growth medium was removed and replaced with 300  $\mu$ L of the medium with 10% FBS. The plasmid complex suspension was then added and incubated for 4 h at 37°C. After addition of the 1 mL fresh medium with 10% FBS, the cells were incubated for a further 40 h at 37°C, and assessed for transgene expression by a luciferase assay kit (Promega). Protein content in the lysate was also analyzed by a protein assay kit (Bio-Rad, Hercules, CA).

Transfection with the complex containing a small amount of PEG-ACs was also attempted. Plasmid/PEI/PEG-AC ternary complex was prepared as follows. To the solution of plasmid (1.25  $\mu$ g in 12.5  $\mu$ L water), PEG-AC (1.25  $\mu$ g in 6.25  $\mu$ L water) and linear PEI (1.3  $\mu$ g in 12.5  $\mu$ L water) were added in this order. After the complex was allowed to stand for 30 min, double-concentrated PBS (50  $\mu$ L) was added.

After the transfection period, the amount of DNA complex remaining in the supernatant was measured as follows. The supernatant (300  $\mu$ L) was collected from each well, and the same volume of 25% NaCl was added. After incubation overnight at 37°C, DAPI (0.76  $\mu$ g in 1200  $\mu$ L water) was added, and the concentration of the DNA was determined by measuring the fluorescence intensity at 461 nm (Ex = 358 nm).

## Model and Monte Carlo simulation

Our simulation model was aimed at understanding the possible spatial correlation of a polycation and a polyampholyte (or a polyanion) around the local segment in a long-chain DNA, shorter than the DNA persistent length. The starting point of our modeling was to modify the well-studied cell model in such a way that the rodlike segment was considered as a short charged cylinder and was confined within an eccentric cylindrical simulation cell. When the monomer density around the rodlike segment is high, the forces around the radial direction due to surrounding DNA segments are basically cancelled. This picture is analogous to modeling polymer melts as a polymer confined in a tube due to significant force cancellation around the tagged chain in the radial direction (17). Hence, this model is applicable to a local segment of DNA either in a loosely compacted structure or within the highly compact ordered structure of a long DNA chain.

In this model, the correction of the long-range tail arising from Coulomb potential beyond the finite simulation cell was implemented along the axis of the segment, just like the infinitely long charged chain of a DNA in the cell model. Even though the infinitely long charged cylinder was used here, under high salt concentration as in our experimental condition, the length-scale of electrostatic potentials was limited around the length of the cylindrical simulation cell. In other words, the remaining DNA chain beyond the simulation cell had little contribution in our system. As a matter of fact, such a model allowed us to reduce the dimension of the simulation cell to a cylindrical cell subjected to the simple one-dimensional periodical boundary condition. More details are given below.

In the calculation, a short DNA segment (length ( $L$ ) =  $18 R_{\text{DNA}}$ ) was modeled as part of a long charged cylinder with radius  $R_{\text{DNA}}$  (10 Å), and polycation and polyampholyte (or polyanion) are confined within a concentric cylindrical simulation cell of radius  $R_{\text{cell}}$  ( $12 R_{\text{DNA}}$ ), which is similar to the cell model except that particles are not allowed to penetrate the cylindrical wall of the simulation cell. Note that a schematic of this model is shown below (see Fig. 8 A). It is known that the conformation of polyanions like polyanions is more elongated due to electrostatic repulsion among charged monomers (18). Hence, polyanions were modeled as shish-kebab rods containing six tangent charged hard spheres of diameter  $\sigma = R_{\text{DNA}}$  aligned along the same molecular axis. Here, each charged bead in the polyanion carried  $f_- = -1$  monomer charge (i.e., a total charge of  $-6$ ), equivalent to the linear charge density 0.1 Coulomb/Å. This charge density is slightly smaller than that of a DNA under counterion condensation ( $\sim 0.15$  Coulomb/Å (19)). The linear charge density was thus chosen to illus-

trate that even though the linear charge density of a polyanion is increased to near the level of the effective linear charge density of a DNA in salt solution, its effectiveness in dissociation of DNA/polycation complexes remains low. For polycations, each of six charged beads in the shish-kebab chain had a monomer charge of  $f_+ = +0.5$ , with a total charge equal to  $+3$ .

Both polycation and polyampholyte were also modeled as shish-kebab rods, but polyampholytes were constituted of two distinct blocks of opposite charges. In an anionic polyampholyte, there were four consecutive negatively charged beads and two positively charged beads in a chain molecule, by assuming that the charge magnitude of each bead is  $|f_{\pm}| = 3$ . These monomer charges allowed the anionic polyampholyte to form a net linear charge density (or a net total charge) identical to that of the above polyanion. In cationic polyampholytes, the magnitude of each monomer charge stayed the same as that of the anionic polyampholyte, except that the sign of each monomer charge was changed. Since polyampholytes may have more complex conformational behavior than homopolyelectrolytes (20), these diblocked polyampholyte models are greatly useful for exploring the effect of the net dipole embedded in a polyampholyte chain (15). With this model, we were able to combine the effects of dipole and net charge in cationic and anionic polyampholytes.

Monte Carlo simulation was used to describe the thermodynamics of the above model, and the mobile polycation and polyampholyte (or polyanion) underwent random rotation and translation to sample their configurations around DNA. A new configuration was accepted based on the standard Metropolis criterion in the Monte Carlo method. Here, we compared three systems in which the DNA/polycation complex blended with a cationic polyampholyte, an anionic polyampholyte, and a polyanion. These ionic particles interacted through the electrostatic interaction at the Debye-Hückel level with the reduced inverse screening length  $\kappa R_{\text{DNA}} = 0.8$  to model the high salt concentration. In this condition, the strong electrostatic screening reduces the lengthscale of the Coulomb interaction. In the simulation, the inverse Debye screening length ( $1/\kappa = 1.25 R_{\text{DNA}}$ ) means that the Coulomb interaction roughly decreases  $>99.9\%$  from the center of the simulation cell to its boundary along the DNA axis ( $18 R_{\text{DNA}}$  for cell length). Namely, even though the infinity long chain DNA was used in the model, the interactions among macroions diminished within the length of the simulation cell. Nevertheless, the Ewald summation was applied to account for any residual long-range tail in the screened Coulomb interaction as in Shew et al. (15).

To better understand the spatial correlation among these three ionic particles, the monomer radial distribution,  $P_{\text{mm}}(r)$ , of polycation and polyampholyte (or polyanion) around DNA was first examined. In addition, we investigated a distribution function,  $P(r_c, \cos \theta_c)$ , of two variables ( $r_c$  and  $\theta_c$ ), as defined in the inset of Fig. 8 A, where  $r_c$  is the length of the vector measured from the center of mass of the polyampholyte (or polyanion) to the center of mass of the polycation, and  $\theta_c$  is the angle between the vector  $\mathbf{r}_c$  and the vector perpendicular to the DNA axis measured from the center of mass of the polyampholyte (or polyanion), as defined in Fig. 8 A. In the limiting case where  $\cos \theta_c = 1$ , the polycation (or DNA) was situated halfway between the polyampholyte and DNA (or polycation), and when  $\cos \theta_c = -1$ , the polyampholyte (or polyanion) was halfway between the other two ionic particles.

## RESULTS AND DISCUSSION

PEG-ACs with various  $\text{NH}_2/\text{COOH}$  ratios were synthesized by an addition reaction of the mercaptans to the double bond of poly(allyl glycidyl ether-co-ethylene oxide). Completion of the reaction was confirmed by  $^1\text{H}$  NMR spectra, where no residual allyl group was detected.

The addition of PEG-ACs did not decompose the DNA/PEI complex nor release free DNA molecules, but afforded DNA/PEI/PEG-AC ternary complexes with various surface potentials.  $\zeta$ -Potential of the plasmid/PEI binary complex

prepared at P/N = 1:8 was +41.1 mV. Addition of PEG-ACs changed the value depending on the  $\text{NH}_2/\text{COOH}$  ratio of the polyampholytes. PEG-ACs with excess carboxyls recharged the preformed plasmid/PEI complexes to negative, whereas amine-excess PEG-ACs resulted in positively charged ternary complexes (Figs. 1 and 2). The sizes of the complexes were ~150–300 nm in diameter, except in the case of PEG-AC(37:63), which had larger diameters (>400 nm), most probably due to the aggregative property of the particles with almost neutral surface potentials (Fig. S1 in the Supporting Material).

A fluorescence anisotropy assay was conducted for DAPI associated with DNA molecules in the complex to analyze the loosening effect of PEG-ACs on the DNA complex. When DNA was compacted by PEI into a globular state, the fluorescence anisotropy of DAPI bound to the DNA molecule was increased to >10%. Cationic PEG-ACs ( $\text{NH}_2/\text{COOH} > 1$ ) had little influence on the anisotropy value. On the other hand, an addition of the anionic PEG-ACs ( $\text{NH}_2/\text{COOH} < 1$ ) to the DNA/PEI complexes diminished the anisotropy. The diminishing range was dependent on the  $\text{NH}_2/\text{COOH}$  ratios of PEG-ACs, and those with  $\text{NH}_2/\text{COOH} = 32:68$  or  $37:63$  showed the highest decreasing levels (Fig. 3 B). Since DAPI in the double-strand DNA has a very short lifetime (2.20 ns), the polarization measurement of the fluorophore seems not to reflect the mobility of the whole-complex particles but should link with the motion of the DNA segment to which it is attached. The decrease in the anisotropy of DAPI molecule thus would be attributed to the swelling of

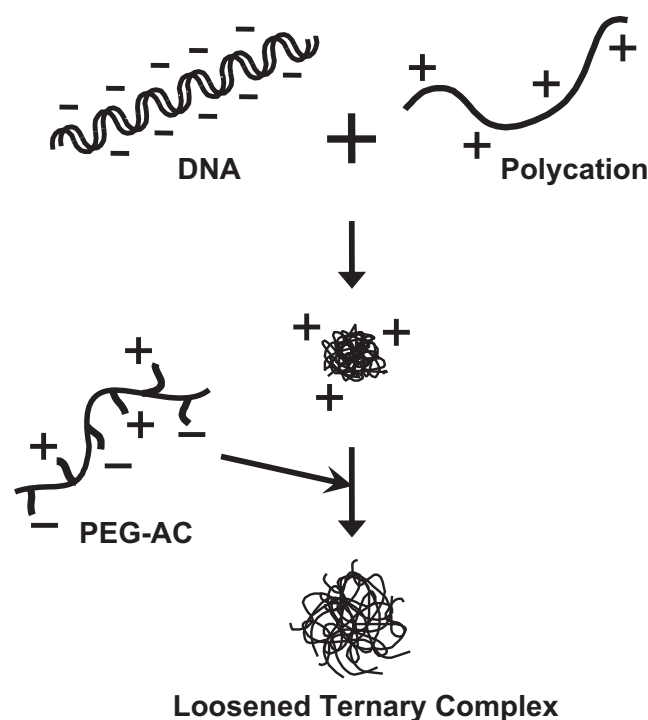


FIGURE 1 Loosening of DNA/polycation complex by amphoteric PEG derivative (PEG-AC).

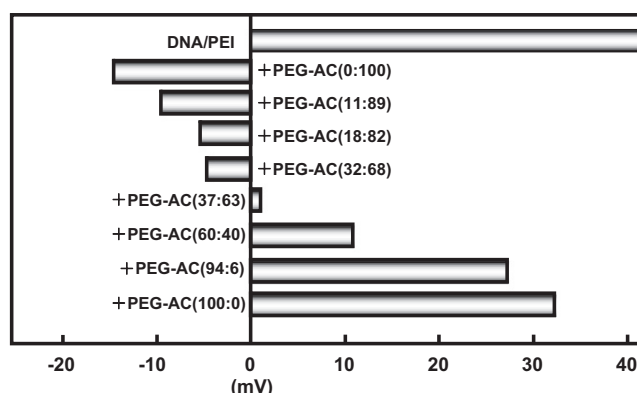


FIGURE 2 ζ-Potential of DNA/PEI/PEG-AC complexes.

the particles and the relaxing of the DNA molecule in the complex to depolarize the bound fluorophore.

As shown in the time-resolved fluorescence anisotropy decay profiles (Figs. 3 B and 4), the amphoteric polymers with  $\text{NH}_2/\text{COOH} = 32:68$  rapidly depolarized the fluorescence, whereas the polyanion, PEG-AC(0:100), bearing only anionic pendants, slowly decreased the anisotropy. This finding suggested that polyampholytes could loosen the DNA/polycation complex through an efficient mechanism different from that of the polyanion.

The effect of the order of addition of PEI and PEG-AC to DNA on the loosening efficiency was then examined systematically. As soon as PEI was added to the mixture of DNA and PEG-ACs, the complexes formed had lower anisotropy than those without the PEG-ACs. However, the time-resolved anisotropy profiles were different from those obtained in the postaddition study. The anisotropy of the ternary complexes was low from the inception of mixing, and any significant change in anisotropy value was not observed, indicating that the complex was formed on PEI addition in the equivalent state. The  $\text{NH}_2/\text{COOH}$  ratio in PEG-AC showed little impact on the anisotropy value. If PEG-AC was added after the formation of DNA/PEI complex, PEG-AC should peel off the preformed polyion complexes along with the change of the structure to a stable equivalent form. A small difference in the  $\text{NH}_2/\text{COOH}$  ratios of the PEG-AC would interfere with the reforming ability. On the other hand, PEG-ACs pre-added to DNA did not need to peel off the PEI/DNA binding to penetrate into the complex. The  $\text{NH}_2/\text{COOH}$  composition in the polyampholyte would thus not significantly affect the loosening degree of the resulting complexes.

Transcriptional activity of the DNA/PEI complex with or without addition of PEG-ACs was examined with RNA polymerase (from *E. coli*) by an in vitro system without cells. After incubation at 37°C for 2 h, the transcription reaction was stopped by buffer containing phenol and EDTA, and mRNA was analyzed. The naked plasmid showed a high yield of RNA, with 18 μg of RNA detected, whereas the reaction with plasmid/PEI binary complex at P/N = 1:8 produced only 1.6 ng of RNA. Although cationic PEG-ACs did not



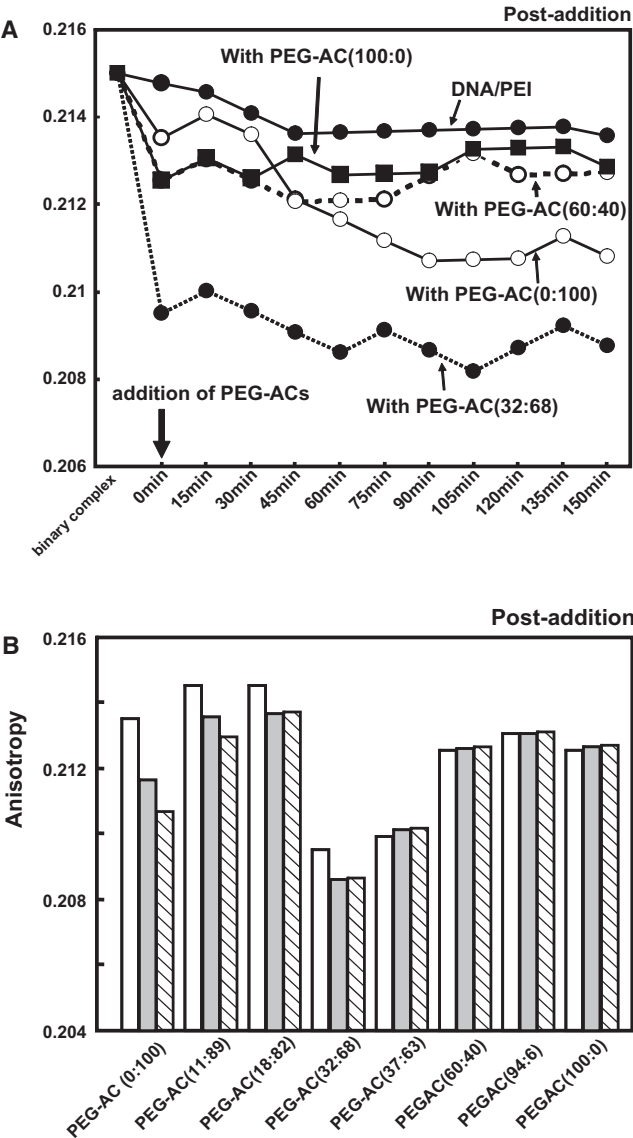


FIGURE 3 Effect of postaddition of PEG-ACs on the fluorescence anisotropy of DAPI bound to DNA molecule. (A) Typical time course of change in fluorescence anisotropy after addition of PEG-ACs. (B) Influence of amino/coxyl ratio on the anisotropy.

improve the mRNA production, an addition of anionic PEG-ACs to the plasmid/PEI complex enhanced the production of RNA. The maximum activity was reached when the composition of anionic groups in an anionic polyampholyte approached 70%, and the RNA production increased up to 31 ng (Fig. 5 A). PEG-AC with  $\text{NH}_2/\text{COOH} = 32:68$ , which showed the highest loosening effect on the DNA/PEI complex in the anisotropy study, again gave the highest effect on the production of mRNA, indicating the effectiveness of such a complex-unfolding agent in improving transcription efficiency. The effect of preaddition of PEG-AC on the transcriptional efficiency of the resulting ternary complexes is shown in Fig. 5 B. Anionic PEG-AC again apparently enhanced the production of mRNA, though cationic PEG-ACs showed

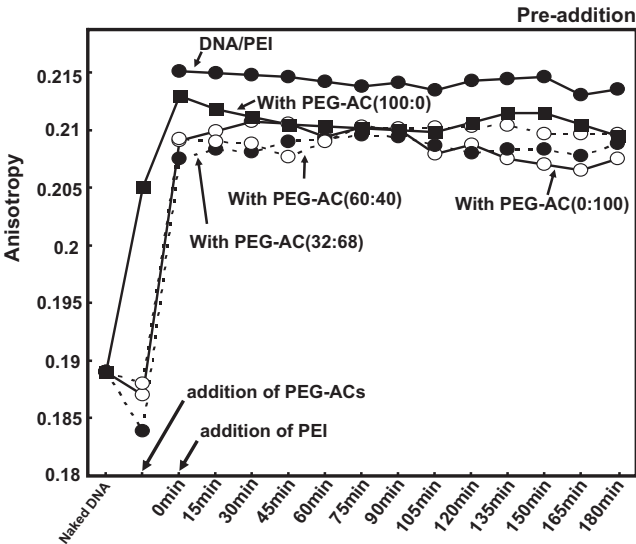


FIGURE 4 Effect of preaddition of PEG-ACs on the fluorescence anisotropy of DAPI bound to DNA molecule.

an insignificant improvement in the efficiency. The detailed composition of the anionic PEG-ACs exhibited little difference, unlike the case with postaddition.

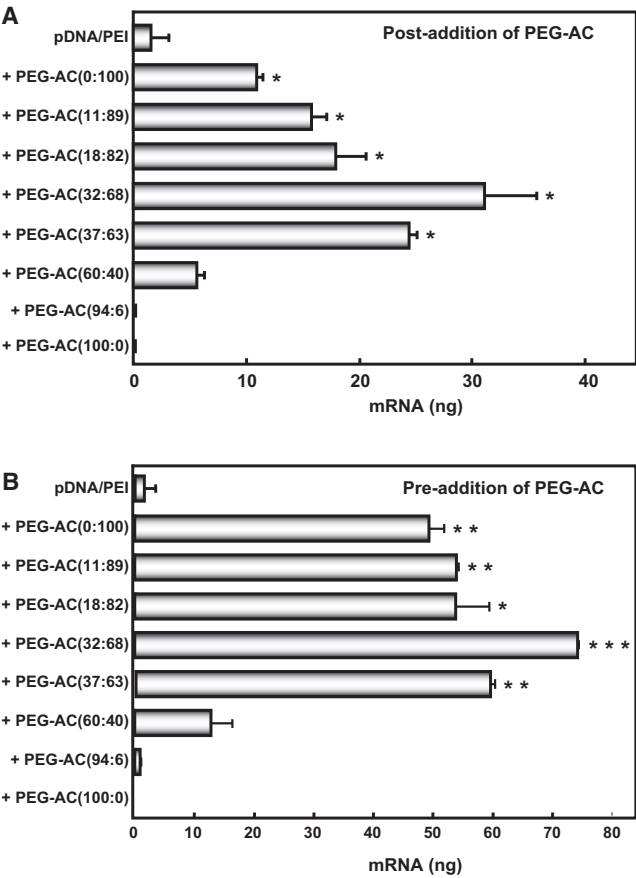


FIGURE 5 Improvement of transcription efficiency by (A) postaddition and (B) preaddition of PEG-ACs to DNA/PEI complexes. Significant difference against DNA/PEI binary complex: \* $P < 0.5$ , \*\* $P < 0.1$ , \*\*\* $P < 0.01$ .

It was noted that the kinetic effect was indeed present in such a way that the transcriptional activity for the process with preaddition of anionic polyampholytes increased the efficiency of transcription activity differently. Nevertheless, the kinetic effect is somehow secondary, because the finding that anionic polyampholytes are more effective than cationic polyampholytes and polyanions in promoting transcriptional activity was independent of addition processes. In other words, thermodynamics is essential to provide physical insights into these systems that can be studied from the aspect of equilibrium statistical mechanics.

Transfection efficiency of the ternary complexes with PEG-ACs, performed in a similar way to the transcription experiments, was examined on the cultured B16 cells or CHO cells. In view of the negative charge of the cell surfaces, like-charge complexes with anionic PEG-ACs seem to have a disadvantage for the gene transfection. However, they showed gene-expressing activity in both cells higher than that seen in the original DNA/PEI binary complex (Fig. 6, A and B). High amounts of PEG-ACs caused the charge to alternate, as shown in Fig. 2. The surface charge of the complex may affect the transgene expression efficiency. Such abundance of polycation may cause side effects such as membrane

damage. The transfection was thus performed with small amounts of PEG-ACs to avoid too much recharging of the complex charge. At a mixing ratio of DNA/PEI/PEG-AC = 1:1.6:1 in weight, all the complexes were positively charged (17–29 mV), and those for which PEG-ACs represented >30% of the amino group showed almost the same surface potentials (27–29 mV). Such a small amount of the anionic PEG-ACs effectively enhanced luciferase production, whereas the same weight of cationic PEG-ACs showed little improvement (Fig. 6 C). Among the anionic PEG-ACs, the complex with PEG-AC(32:68) again presented the highest transfection efficiency, and the extragene expression of the DNA/PEI/PEG-AC(32:68) ternary complex was >10 times higher than the DNA/PEI complex without PEG-AC ( $P < 0.01$ ). Cell-uptake efficiency of the DNA/polycation/PEG-AC complexes was examined by analyzing the DNA concentration in the supernatant after the transfection. The ratio of the complex precipitated onto the cells was dependent on the absolute value of their surface potential. The precipitated ratio of the complexes with low absolute value of  $\zeta$ -potential ( $<5$  mV) was 53–59%, whereas those for the complexes with high absolute values ( $>10$  mV) were 40–48%. The complexes with low absolute potential would aggregate during the transfection period, accelerating the precipitation. The precipitating ratio did not depend on whether the charge was plus or minus, and it was not much different between the positively and negatively charged complexes. Consequently, in all cases  $50 \pm 10\%$  of the complex was precipitated, and the difference among the PEG-ACs was much smaller than that seen in the transfection results ( $>10$  times). Although the complexes taken up inside the cells could not be distinguished from those only adhered to the cell surfaces, uptake efficiency would not account for the significant difference in the gene expression, and the high gene expression with anionic PEG-ACs would thus be most likely attributed to their improved transcriptional efficiency.

The complex with PEG-AC(32:68) again presented the highest transfection efficiency among the anionic PEG-ACs, though the difference is not so significant as seen in the transcription experiments. High accessibility to the DNA in the complex with PEG-AC(32:68) may also invite a high degradability of the DNA molecule by DNase in the endosome. Nevertheless, the extragene expression of the DNA/PEI/PEG-AC(32:68) ternary complex was >10 times higher than that of the DNA/PEI complex without PEG-AC ( $P < 0.05$ ) and more than three times higher than that of the DNA/PEI/PEG-AC(0:100) complex ( $P < 0.01$ ).

Monte Carlo simulation was performed to explore the loosening mechanism of DNA/polycation complexes by polyampholytes in thermodynamic limit. In the simulation, the spatial correlation among these three ionic particles was first examined through the monomer radial distribution,  $P_{mm}(r)$ , of polycation and polyampholyte (or polyanion) around DNA (Fig. 7). In addition, we investigated a distribution function,  $P(r_c, \cos \theta_c)$ , of two variables ( $r_c$  and  $\theta_c$ ) (Fig. 8).

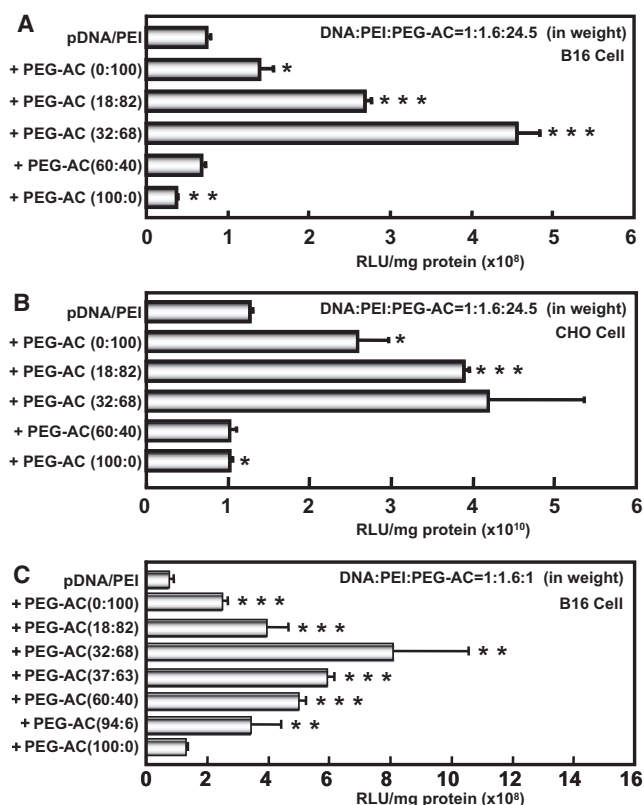


FIGURE 6 Effect of PEG-ACs on gene expression efficiency on (A) B16 cells: DNA/PEI/PEG-AC = 1:1.6:24.5 (in weight); (B) CHO cells: DNA/PEI/PEG-AC = 1:1.6:24.5 (in weight); and (C) B16 cells: DNA/PEI/PEG-AC = 1:1.6:1 (in weight). Significant difference against DNA/PEI binary complex: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

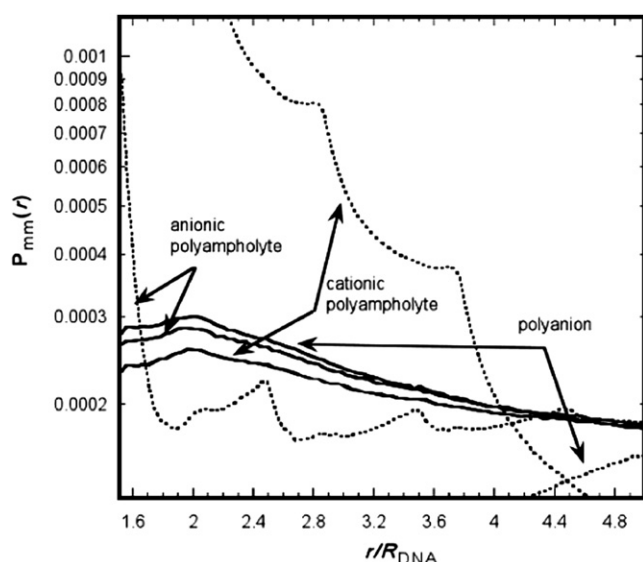


FIGURE 7 Plot of  $P_{mm}(r)$  of polyampholyte (or polyanion) and polycation, denoted by dotted line and solid lines, respectively, when the DNA/polycation complex was mixed with an anionic polyampholyte, a cationic polyampholyte, and a polyanion, as indicated.

Fig. 7 showed that the cationic polyampholyte exhibited the highest probability to distribute around DNA, due to the high positive charge density in its positively charged block. Despite its net negative charge, the anionic polyampholyte also distributed around DNA preferentially due to the considerable dipole moment of the polymer, but its distribution was not as pronounced as that of the cationic polyampholyte. As for the polyanion, the negatively charged polyanion was strongly repelled by the like-charge DNA, resulting in the low distribution around the DNA surface. Furthermore, we found that the distribution of polycation around DNA was sensitive to the type of ionic polymers selected as the third component. The distribution of polycation near DNA increased in the order cationic polyampholyte, anionic polyampholyte, and polyanion. The competition between the cationic polyampholyte and the like-charge polycation was a possible cause for the polycation showing the lowest distribution around DNA among the three cases. In contrast, the highest polycation distribution near DNA was observed while blending with a polyanion. This implied that the polyanion charge density in our model study was insufficient to break the complex of polycation and DNA. For the anionic polyampholyte case, the polycation distribution was between those of the other two cases, even though it had the same total charge as the polyanion. This result indicated that the charge arrangement and charge density along a polyampholyte chain would serve as additional parameters to influence the interaction between DNA and polycation.

To better understand the interplay among different ionic particles, the distribution function  $P(r_c, \cos \theta_c)$  defined in Fig. 8 A was calculated. For the cationic polyampholyte in Fig. 8 A, the global maximum was found between  $\theta_c = 0^\circ$

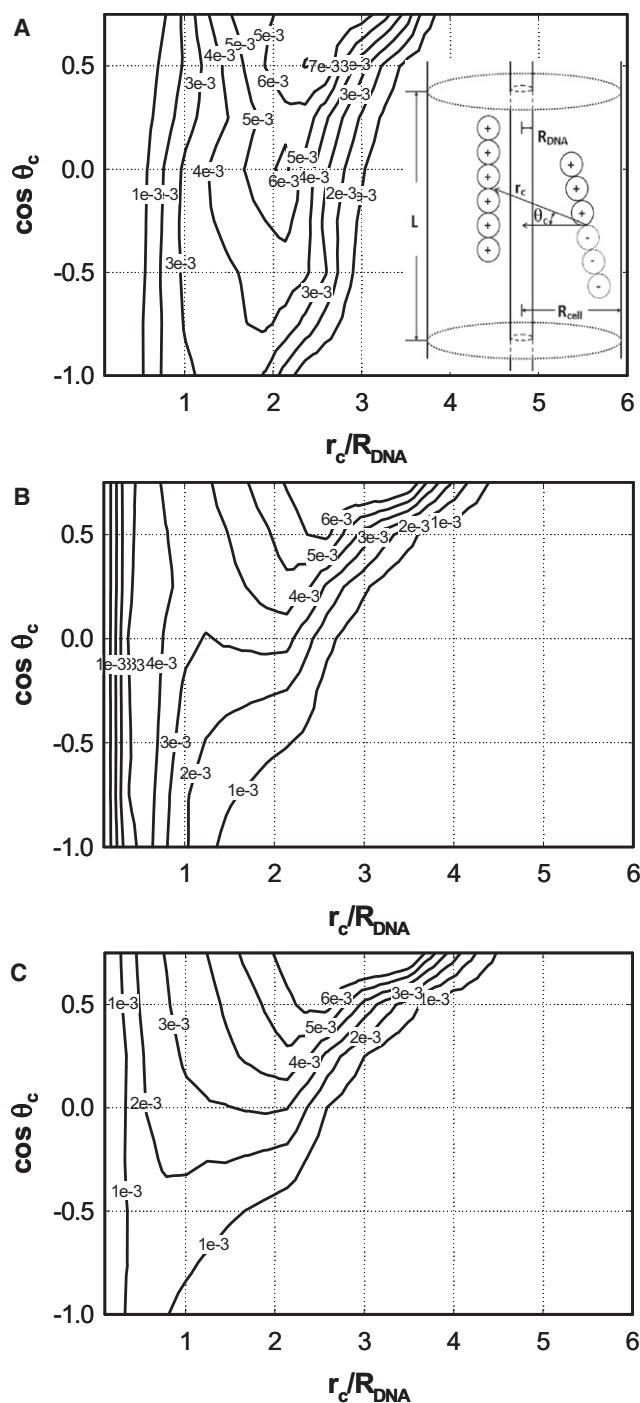


FIGURE 8 Contour plot of  $P(r_c, \cos \theta_c)$  in the presence of (A) a cationic polyampholyte, (B) an anionic polyampholyte, and (C) a polyanion.

and  $60^\circ$  ( $\cos \theta_c = 1/2$ ) as well as around  $r_c = 2.5 R_{DNA}$ , whereas for anionic polyampholyte and polyanion, the global maxima were close to each other and shifted to near  $\cos \theta_c = 1$  ( $\theta_c = 0^\circ$ ) and  $r_c = 3 R_{DNA}$ . In the cationic polyampholyte case, both polycation and polyampholyte tended to avoid aligning in the same direction at the global maximum, which would maximize their attraction to DNA.

Meanwhile, compared to the anionic polyampholyte and the polyanion (Fig. 8, *B* and *C*), the cationic polyampholyte had a narrower  $r_c$  distribution at around  $\cos \theta_c = 1$  ( $\theta_c = 0^\circ$ ), indicating that when a cationic polyampholyte aligns with DNA and the polycation is not in the middle position, the configuration becomes less favorable. For anionic polyampholytes and polyanions, at their global maxima, the anionic polyampholyte (or polyanion) aligns along with the DNA and polycation, but is not situated between these two ionic species. This configuration leads to a possible route to minimize the repulsion between DNA and anionic polyions (polyanions and anionic polyampholytes). Also, it creates a pathway for dissociation of DNA/polycation complexes, especially when the negative charge density of anionic polymers is large enough to drive polycations away from the DNA surface (14).

Unlike anionic polyampholytes and polyanions, cationic polyampholytes displayed a greater probability of being in the middle position between DNA and polycation, in that they exhibited broad distribution with a wide range of  $r_c$  near  $\theta_c = 180^\circ$  ( $\cos \theta_c = -1$ ) (Fig. 8 *A*). Based on this finding together with the data shown in Fig. 7, we argue that the cationic polyampholyte in our model replaces the polycation and becomes strongly bound to DNA. From the standpoint of experiment, strong binding impedes transcriptional activity, because the positive charge of the complex with cationic PEG-ACs would interfere with the association of DNA with the enzyme. The RNA synthesis process consists of three steps: association, initiation, and polymerization. An association of DNA and RNA polymerase in the first step is known to be inhibited by high ionic strength (21). This indicates the importance of the electrostatic interaction between the enzyme and the template DNA. In Fig. 2, the ternary complexes with PEG-ACs having a higher ratio of amine are shown to have the positive surface potential, which would interfere with the association between DNA and the enzyme and cause the low production of mRNA with those complexes seen in Fig. 5, *A* and *B*.

For anionic polyampholytes (Fig. 8 *B*), the distribution near  $\theta_c = 180^\circ$  ( $\cos \theta_c = -1$ ) became narrower than that for cationic polyampholytes (Fig. 8 *A*), but wider than that for the polyanion (Fig. 8 *C*). It is notable that the net negative charge of the anionic polyampholyte was unfavorable for binding with DNA in principle, but based on the data in Fig. 7, the anionic polyampholyte remained active to bind with DNA, since its distribution probability near DNA was still quite high compared to that of polyanions. Meanwhile, in contrast to the case of polyanion (Fig. 7), the polycation associated with the anionic polyampholyte had a lower probability of being near DNA, meaning that the polycation has a weaker affinity to bind with DNA. These features provide a pathway to form electrostatic bridging, arising from a dipolar polyampholyte to connect the DNA and a polycation with its oppositely charged segments. This mechanism enables the creation of some space around DNA for transcriptional

activity. In conjunction with the distribution around  $\theta_c = 0^\circ$  and  $180^\circ$ , these results suggest that anionic polyampholytes induce at least two pathways to enhance dissociation of a DNA/polycation complex, including electrostatic bridging ( $\theta_c = 180^\circ$ ) to bridge both polycation and DNA and weakening polycation adsorption on the like-charge DNA surface ( $\theta_c = 0^\circ$ ) by its high negative charge density in one block. Such dual dissociation mechanisms may facilitate a higher efficiency of transcriptional activity, as observed in the experiment. In the case of polyanions, the distribution near  $\theta_c = 180^\circ$  became too low to have significant electrostatic bridging. As such, the mechanism for loosening the DNA/polycation complexes occurs through competing association of the polyanion (PEG-AC(0:100)) (a higher probability distribution around  $\theta_c = 0^\circ$ ) that could weaken the polycation adsorption on the DNA molecule and allow the DNA segment to become mobile enough to depolarize the bound fluorophore. However, it would not so effectively bring about the accessibility of the enzyme as would the creation of space by bridging between DNA and polycation by the anionic polyampholytes. In particular, the polyanions of low charge density were unable to play a significant role in dissociation of the DNA/polycation complexes. Consequently, the mRNA production-enhancing effect of PEG-AC(0:100) was expected to be relatively low compared to that for other anionic PEG-ACs (Fig. 5), despite its high depolarizing effect (Fig. 3). Note that for a further test, the linear charge density of polyampholytes and polyanion was reduced by half (data not shown), and we arrived at the same conclusion. The model study here provides insights into the dissociation process enhanced by anionic polyampholytes.

Both intramolecular compaction and intermolecular assembly might have occurred in our systems by considering the experimental DNA concentration. In view of the reports concerning the compaction of DNA molecules by polycations, the size of the salmon sperm DNA used in the anisotropy study was 30–45 kbp, which is large enough to show the tight compaction through the discrete intramolecular folding transition. In addition, the plasmid DNA used in the other experiments was 7.0 kbp. It is also much larger than the persistent length to give rise to intramolecular compaction. In addition to single chain compaction, plasmid DNA of the same size has been shown to be condensed (or assembled) by PEI into tightly compacted toroid- or rod-shaped complexes with a size ranging of  $55 \pm 12$  nm (22). Meanwhile, the continuous condensation of a circular plasmid (12.5 kbp) induced by spermine was also reported (23). Circular plasmid has a superhelix structure with highly condensed segments, and its conformation is more influenced by condensing agents depending on the condensation conditions. Superiority of the anionic PEG-ACs over the polyanion, PEG-AC(0:100), was more pronounced in the transcription and transfection experiments with plasmid than in the anisotropy results with linear salmon sperm



DNA. Polyampholytes need to penetrate between DNA and PEI to exert their high loosening effect. It should require a larger conformation change on the DNA molecule than the competitive binding of the polyanion to PEI, peeling it off from the DNA. The high sensitivity of the plasmid molecular conformation would invite a higher response to PEG-ACs.

The transcription experiments of the DNA/PEI/PEG-AC ternary complexes strongly reflected the depolarization behavior of the polyampholytes. Cationic PEG-ACs again showed almost no effect on the transcription. However, those complexes with the greater extent of diminished anisotropy tend to have a higher transcriptional efficiency. As a result, the transcription reaction with DNA complexes containing anionic PEG-ACs was shown to yield greater amounts of mRNA. Also, the improved range by postaddition of the anionic PEG-ACs is very sensitive to the composition of PEG-AC, whereas the effects were not so different compared to those by preaddition of the anionic PEG-ACs. When PEG-AC was added to the preformed DNA/PEI complex, PEG-AC could hardly create a space large enough to facilitate the access of RNA polymerase at the inner part of it. Thus, transcription would proceed mainly on the surface DNA molecules. On the other hand, preadded PEG-AC would readily loosen the inner part of the complex, and the DNA inside would be also transcribed. It would cause the higher level of mRNA production with the PEG-AC-preadded complexes. Depolarization of the fluorophore requires not so large a space as enzyme penetration. Thus, anisotropy could be similarly diminished by both pre- and postaddition of PEG-ACs.

Considering the finding that anionic PEG-AC with high fluorescence anisotropy-diminishing property induced highly enhanced transcriptional efficiency, the loosening effect on the tightly compacted DNA complexes by the polyampholytes would facilitate the approach of transcriptional factors to DNA segments in the complex.

PEG-ACs with excess carboxyl groups were also shown to improve the transgene expression level on the cultured cells. These data indicated the importance of improving the transcriptional efficiency to attain a high extragenic expression by a plasmid complex. These findings can be well understood from our theoretical studies.

## CONCLUSION

Transgene expression level of the plasmid/polyethyleneimine complex could be improved by mixing of an amphoteric polymer, PEG-AC, with carboxyls in excess of amines. The polyampholyte would thus be seen as a novel means of improving multifunctional gene transfection, which gives plasmid complexes stable suspension, protects them against unfavorable interaction with serum components, and enhances their transcriptional and transfectional efficiency. It may introduce a new way to enhance the efficiency of

polycation-mediated gene transfection simply by adding such an activating agent to the vectors. Our theoretical analysis of a simplified model suggests that anionic polyampholytes, in particular, may enhance the dissociation of a plasmid/polyethyleneimine complex with their net dipole and net negative charge, in which the net dipole separates oppositely charged DNA and polycation through electrostatic bridging, and the net negative charge drives the positively charged polyethyleneimine away from DNA.

## SUPPORTING MATERIAL

A figure is available at [http://www.biophysj.org/biophysj/supplemental/S0006-3495\(09\)06094-9](http://www.biophysj.org/biophysj/supplemental/S0006-3495(09)06094-9).

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